EXHIBIT A

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Monoclonal antibodies to glycoprotein B differentiate human herpesvirus 6 into two clusters, variants A and B

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The distribution of glycoprotein B (gB) among different human herpesvirus 6 (HHV-6) strains was analysed with a panel of three monoclonal antibodies (MAbs) derived from mice immunized with U1102-infected lymphocytes. MAb 2D9 reacted specifically by immunofluorescence and immunoprecipitation with the U1102 and GS isolates, and failed to react with Z29 and the variant B strains Hashimoto and SF. In addition, Z29, Hashimoto and SF gB had a lower M_r than U1102 and GS gB. MAb 2D9 also failed to react with the exanthem subitum isolate CV, included in this study as an as yet poorly characterized isolate. Consistent with this result, CV failed to react with the variant A-specific MAb to gp82-105 and behaved as a variant B virus even with respect to the diagnostic *Hin*dIII endonuclease restriction cleavage site located in a fragment hybridizing to the pZVH14 probe. By contrast with MAb 2D9, MAbs 2B9 and 2D10 reacted with all of the isolates tested, strengthening the argument that they have common epitopes. Based on the antigenic and M_r specificities of gB, the HHV-6 isolates tested were arranged into two non-overlapping clusters, which closely parallel the variant A and B strain groups, defined previously by several criteria, including restriction endonuclease polymorphism, antigenic variations, growth in *in vitro* cultures and sequence analyses.

Numerous isolates of the T lymphotropic human herpesviruses designated human herpesvirus 6 (HHV-6) have been described (Downing *et al.*, 1987; Lopez *et al.*, 1988; Tedder *et al.*, 1987). The first strains, GS, U1102 and Z29, were derived from the peripheral blood of AIDS patients (Salahuddin *et al.*, 1986; Downing *et al.*, 1987; Lopez *et al.*, 1988). Subsequently, viruses similar to Z29 were derived from cases of exanthem subitum and shown to be the causative agents of the disease (Yamanishi *et al.*, 1988). Additional isolates were obtained from a number of pathological conditions as well as from healthy individuals (Pellett *et al.*, 1992). The U1102, GS and Z29 strains show cross-reactivity, a genome with an overlapping structure, identical base composition and size, and extensive cross-hybridization (Pellett *et al.*, 1992). The genomes of U1102 and GS and that of Z29 were found early to differ with respect to a *Hin*dIII restriction endonuclease site (Becker *et al.*, 1989; Jarret *et al.*, 1989; Josephs *et al.*, 1988) located in a fragment hybridizing to the pZVH14 probe (Josephs *et al.*, 1986). Subsequently, the HHV-6 strains were differentiated into two clusters on the basis of several criteria, particularly restriction endonuclease cleavage pattern, reactivity to monoclonal antibodies (MAbs), growth in various cell lines *in vitro* (Frenkel *et al.*, 1990; Wyatt *et al.*, 1990; Aubin *et al.*, 1991; Schirmer *et al.*, 1991; Ablashi *et al.*, 1991). The two clusters were designated variants A and B, *ad interim*, until more evidence is gained for the basis of the clustering and its biological and epidemiological significance (Ablashi *et al.*, 1993). GS and U1102 belong to variant group A, which includes adult-derived strains. Z29 is the prototype of variant group B, which includes isolates derived from children with roseola infantum. MAbs to gp82-105 and to p180 (Balachandran *et al.*, 1989) react selectively with variant A strains; MAb OHV1 (Okuno *et al.*, 1992) reacts specifically with variant B viruses (Aubin *et al.*, 1993). Comparative sequence analyses on restricted portions of the genomes of the two variant prototypes and of several strains showed that variations between variant A and B strains ranged from 4 to 6%, and variations within each group ranged from 0·2% to between 2 and 3% (Aubin *et al.*, 1993; Gompels *et al.*, 1993). These findings confirmed the very close relationship among the strains and strengthened the hypothesis of clustering of the HHV-6 isolates into two variant groups.

Earlier, our laboratory reported that MAb 2D10 reacts with a glycoprotein initially designated gp112 (Foà-Tomasi *et al.*, 1992), and subsequently shown to be the homologue of glycoprotein B (gB) encoded by HHV-

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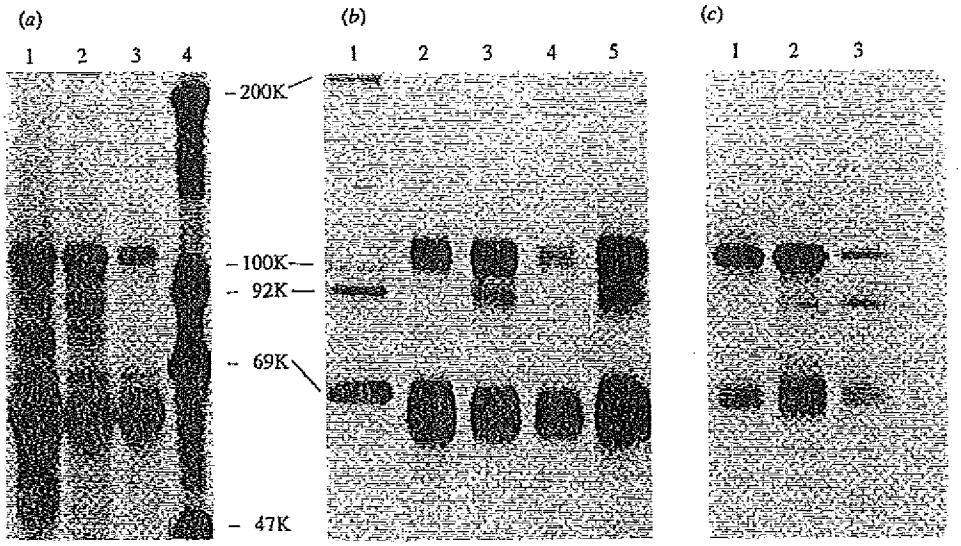

Fig. 1. Characterization of MAbs 2D9 and 2B9 and of the reacting glycoprotein. (*a*) Electrophoretic profile of the proteins immunoprecipitated by MAbs 2D10 (lane 1), 2D9 (lane 2) and 2B9 (lane 3) from lysates of HHV-6 (U1102)-infected [^{35}S]methionine-labelled CBLs. (*b*) Electrophoretic profile of the proteins immunoprecipitated by MAbs 2D10 (lane 2), 2D9 (lane 3), 2B9 (lane 4) and MAb anti-gp116-64-54 (lane 5) from lysates of HHV-6 (U1102)-infected CBLs labelled with 6-D-[^{3}H]glucosamine. (*c*) Electrophoretic profile of protein immunoprecipitated by MAb 2D9 from lysates of HHV-6 (U1102)-infected CBLs pulse-labelled with [^{3}H]glucosamine. Cells were harvested immediately after the pulse (lane 1) or after a 2 h chase (lane 2) in non-radioactive medium. Lane 3, cells were continuously labelled with [^{3}H]glucosamine for 12 h. The migration positions of radioactive M_r markers are shown in (*a*) lane 4 and (*b*) lane 1.

6 (Ellinger *et al.*, 1993). U1102- and GS-encoded gB were found to differ from the Z29 gB in that the former consist of three glycosylated polypeptides of M_r 112000, 62000 and 58000, whereas the latter consists of three glycosylated polypeptides of M_r 102000, 59000 and 50000 (Foà-Tomasi *et al.*, 1992). For both U1102 and GS gB and Z29 gB the two lower M_r polypeptides appear to be generated by proteolytic cleavage of the higher M_r polypeptide (Foà-Tomasi *et al.*, 1992). In this paper we derived two further MAbs to U1102 gB and examined the antigenic variations in the gB of a number of HHV-6 strains. We report that MAb 2D9 reacts specifically with GS and U1102 and does not react with Z29, the Hashimoto (Yamanishi *et al.*, 1988) and SF (Levy *et al.*, 1990) strains previously classified as B variants (Schirmer *et al.*, 1991; Chandran *et al.*, 1992), nor with CV, an as yet poorly characterized isolate derived from a roseola infantum case (Portolani *et al.*, 1990). Our findings indicate that gB, a highly conserved glycoprotein within the *Herpesviridae* family, undergoes antigenic variations among the HHV-6 isolates tested. The clustering based on antigenic variations of gB parallels closely the segregation into variant groups A and B.

MAbs 2B9 and 2D9 were derived from a mouse immunized with U1102-infected cord blood lymphocytes (CBLs), as described previously for MAb 2E4 (Foà-Tomasi *et al.*, 1991). The hybridoma secreting MAb 2D10 was derived independently of those secreting MAbs 2B9 and 2D9. All hybridomas were cloned twice. The culture of HHV-6 in CBLs has been described (Foà-Tomasi *et al.*, 1991). The SF isolate was grown on peripheral blood lymphocytes. Labelling conditions were as follows. Infected CBLs were labelled for 12 h with [^{35}S]methionine (1300 Ci/mmol) at a concentration of 50 μCi/ml of medium containing 1/20 of the usual concentration of unlabelled methionine or labelled for 24 h with 6-D-[^{3}H]glucosamine (33 Ci/mmol), at a concentration of 100 μCi/ml of medium containing one-third of the usual concentration of glucose. Pulse labelling was with [^{3}H]glucosamine (500 μCi/ml of medium containing no glucose) for 1 h. The proteins immunoprecipitated with the indicated MAbs were harvested with Protein A–Sepharose and electrophoretically separated in denaturing polyacrylamide gels, as detailed (Campadelli-Fiume *et al.*, 1988). The gels were soaked in Amplify (Amersham) and the dried gels were exposed to Kodak X-Omat films for fluorography. Indirect immunofluorescence analysis (IFA) of acetone-fixed cells was as detailed (Foà-Tomasi *et al.*, 1991).

Fig. 1(*a*) shows that MAbs 2D9 (lane 2) and 2B9 (lane 3) precipitated three major polypeptides from lysates of HHV-6 (U1102)-infected [^{35}S]methionine-labelled CBLs, whose apparent M_r values in denaturing polyacrylamide gels were 112000, 62000 and 58000, respectively. The electrophoretic profile is indistinguishable from that of gB immunoprecipitated by MAb 2D10 (lane 1). The

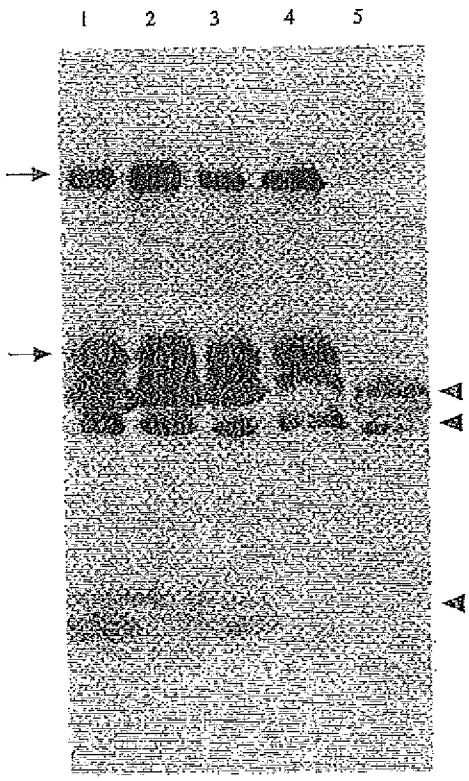

Fig. 2. Radioimmunoprecipitation with MAbs 2B9, 2D10, 2D9 and anti-gp116-64-54 followed by immunoblot analysis of the precipitated proteins with MAb 2D10. Replicate amounts of a lysate made from [^{35}S]methionine-labelled U1102-infected CBLs were immunoprecipitated with MAbs 2B9 (lane 1), 2D10 (lane 2), 2D9 (lane 3) and anti-gp116-54-54 (lane 4) and MAb HD1 to herpes simplex virus glycoprotein D (lane 5), as a negative control. The immunoprecipitated proteins were first subjected to denaturing PAGE, transferred to a nitrocellulose sheet and then allowed to react with MAb 2D10. The reacting proteins were visualized by using an avidin-amplified ABC mouse IgG kit (Vector Laboratories). The staining revealed gB (arrows) as well as the IgG molecules (arrowheads).

electrophoretic profiles of the proteins immunoprecipitated from [^{3}H]glucosamine-labelled HHV-6 (U1102)-infected CBLs by MAbs 2B9, 2D9 and 2D10 were also practically indistinguishable (Fig. 1*b*). The only difference concerned an additional polypeptide, 88000 in apparent M_r, visible in the precipitates using MAb 2D9. To investigate the origin of this polypeptide a pulse–chase experiment was done. As shown in Fig. 1(*c*), the M_r 88000 polypeptide was absent in cells harvested after a 1 h pulse (lane 1), and became apparent in chased cells (lane 2), whereas both the M_r 112000 precursor and the M_r 62000/58000 cleaved polypeptides were already present in cells labelled for 1 h. The results suggest that the M_r 88000 polypeptide originated by a post-translational event, probably by oligosaccharide conversion of one of the two low M_r polypeptides to a higher M_r polypeptide or by a late proteolytic cleavage.

The near identity of the electrophoretic profiles of the proteins immunoprecipitated by MAbs 2B9, 2D9 and 2D10 and by MAb to gp116-64-54 (Universal Biotechnology) (Fig. 1*b*, lane 5) (Balachandran *et al.*, 1989) suggested that the four MAbs react with one and the same glycoprotein. We took advantage of the ability of MAb 2D10 to react with denatured proteins to establish that this is indeed the case. Lysates of HHV-6 (U1102)-infected [^{35}S]methionine-labelled CBLs were immunoprecipitated with MAbs 2B9, 2D9, 2D10 and MAbs to gp116-64-54. The precipitated proteins were separated in denaturing gels, transferred to a nitrocellulose sheet and reacted with MAb 2D10. The ability of MAb 2D10 to react with the glycoproteins precipitated by any of the four MAbs (Fig. 2) indicates that MAbs 2B9, 2D9 and 2D10 all react with gB, and that the glycoprotein designated gp116-64-54 by Balachandran *et al.* (1989) is also gB. Previously, the epitopes reacting with MAbs 2D10 and 2B9 were shown to be present in a plasmid-encoded product containing 79 amino acids from the C-terminal cytoplasmic tail of U1102 gB (Ellinger *et al.*, 1993). The lack of reactivity of MAb 2D10 to the M_r 88000 polypeptide in immunoblots (Foà-Tomasi *et al.*, 1992) favours the view that this polypeptide originates from the N-terminal rather than the C-terminal portion of the molecule. Consistent with this possibility is the observation that the N-terminal half of GS gB is more divergent than the C-terminal portion as compared to Z29 gB (Chou & Marousek, 1992). The reported variations may well be sufficient to allow variations in epitopes, as single amino acid substitutions may account for lack of reactivity to antibody [see as an example MAb-resistant mutations in herpes simplex virus glycoprotein D (Minson *et al.*, 1986)] or for a strain-specific epitope [see for example human cytomegalovirus gH (Urban *et al.*, 1992)]. In addition, based on the ability of MAbs 2D10 and 2B9 to immunoprecipitate both the 62000 and the 58000 M_r polypeptides, and on the strong similarities between Z29 gB (Foà-Tomasi *et al.*, 1992) and the protein recognized by MAb OHV1 (Okuna *et al.*, 1992) it can be assumed that the 62000 and 58000 M_r polypeptides are disulphide-bonded. If this is the case, the absence of the 88000 M_r polypeptide from the proteins immunoprecipitated by MAbs 2D10 and 2B9 would imply that, in the detergent-solubilized state, the 88000 M_r polypeptide lacks the epitope reacting with MAbs 2D10 and 2B9.

In the next series of experiments we investigated whether MAbs 2B9 and 2D9 can detect antigenic variations in gB expressed from various HHV-6 isolates. In addition to U1102, the strains examined were GS, Z29, the Z29-like Hashimoto derived from a case of exanthem subitum (Yamanishi *et al.*, 1988; Schirmer *et al.*, 1991), and the B variant SF, derived in San Francisco

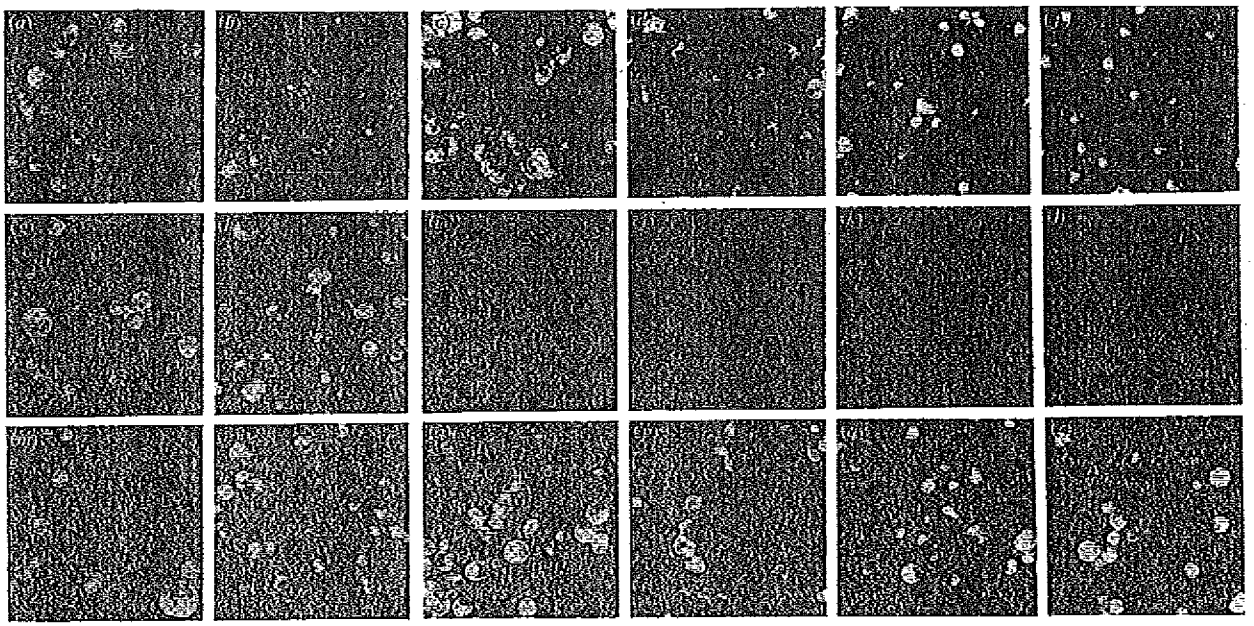

Fig. 3. Immunofluorescence analysis of lymphocytes infected with various HHV-6 isolates and stained with MAbs 2B9 (*a* to *f*), 2D9 (*g* to *l*) and 2D10 (*m* to *r*). Cells were infected as follows: (*a, g* and *m*) U1102; (*b, h* and *n*) GS; (*c, i* and *o*) Z29; (*d, j* and *p*) Hashimoto; (*e, k* and *q*) SF; (*f, l* and *r*) CV.

from the saliva of a human immunodeficiency virus-positive individual (Levy *et al.*, 1990; Chandran *et al.*, 1992). The isolate CV, derived in Italy from a case of exanthem subitum (Portolani *et al.*, 1990), was included as an as yet poorly characterized HHV-6 isolate. By IFA MAb 2D9 reacted with cells infected with the U1102 and GS but failed to react with cells infected with the Z29, Hashimoto, SF and CV isolates (Fig. 3). By contrast, MAbs 2B9 and 2D10 reacted with cells infected with any isolate. The results indicate that MAb 2D9 specifically fails to react with Z29, Hashimoto, CV and SF. MAb anti-gp116-64-54 reacted with all the isolates tested at a 100-fold dilution (data not shown), in agreement with previous data (Wyatt *et al.*, 1990; Ablashi *et al.*, 1991).

Previously, our laboratory reported that Z29 gB has a lower M_r than U1102 and GS gB (Foà-Tomasi *et al.*, 1992). MAbs 2D10 and 2D9 were next tested in an immunoprecipitation assay with the above isolates. The objectives of this experiment were twofold: first, to ascertain whether the lower M_r of Z29 gB is specific to this strain or is a variation common to variant B viruses; second, to ascertain whether the lack of reactivity of MAb 2D9 detected in IFA is reflected in an immunoprecipitation test. Lysates of [^{35}S]methionine-labelled infected CBLs were immunoprecipitated with MAbs 2D10 and 2D9. The pattern of reactivity in immunoprecipitation reflected closely that in IFA, inasmuch as MAb 2D9 precipitated gB from U1102- and GS-infected CBLs, but failed to precipitate any protein from CBLs infected with the Z29, Hashimoto, SF or CV isolates

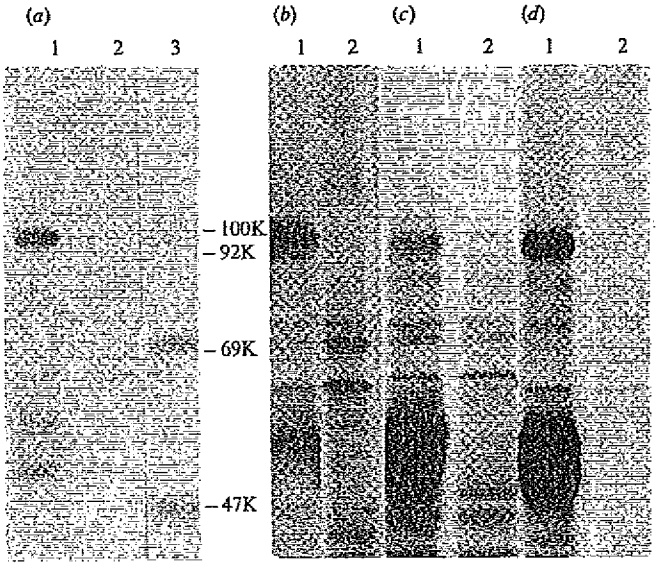

Fig. 4. Radioimmunoprecipitation of gB with MAb 2D10 (lanes 1) and MAb 2D9 (lanes 2) from lysates of CBLs infected with the following isolates: Z29 (*a*), Hashimoto (*b*), SF (*c*) and CV (*d*). The migration positions of the radioactive M_r markers are shown in (*a*) lane 3.

(Fig. 4). MAb 2D10 immunoprecipitated gB from CBLs infected with any isolate (Fig. 4). The electrophoretic profile of the immunoprecipitated proteins revealed that gB expressed by Hashimoto, CV and SF consisted of three polypeptides similar in M_r to those of Z29 gB. Two minor differences were observed. The higher M_r band in Hashimoto gB appeared to consist not of one but of two

polypeptides, of 102000 and 97000 apparent M_r. The higher band in SF gB had an apparent M_r of 98000.

Since CV displayed the same reactivity to MAb 2D9 as Z29, we analysed the isolate for reactivity with the variant A-specific MAb to gp82-105 (Universal Biotechnology) and for the diagnostic variant B *Hin*dIII endonuclease restriction site. CV failed to react with the MAb (data not shown). The 830 bp fragment amplified by PCR according to Aubin *et al.* (1991) was cleaved by *Hin*dIII into two fragments (D. Di Luca & G. Campadelli-Fiume, unpublished), indicating that CV may be classified as a variant B virus with respect to these two parameters.

Earlier, our laboratory identified the gB homologue encoded by U1102, GS and Z29 with the aid of MAb 2D10 (Ellinger *et al.*, 1993). In current studies we derived two additional MAbs (2B9 and 2D9) against HHV-6 gB and examined their reactivity with several HHV-6 strains. The relevant antibody in this study was MAb 2D9, which differs from MAbs 2D10 and 2B9 in its ability to immunoprecipitate an additional 88000 M_r polypeptide and in its specific reactivity with some of the isolates tested. The finding that HHV-6 gB was expressed by all the isolates is in agreement with the notion that gB, along with gH, is highly conserved in the *Herpesviridae* family.

Two co-variations were detected in gB encoded by the isolates tested. One concerned the selective reactivity of MAb 2D9, whose reacting epitope is either absent or modified in Z29, Hashimoto, SF and CV gB. The second variation concerned the lower apparent M_r of Z29, Hashimoto, SF and CV gB, as compared to that of U1102 and GS gB. The differences are likely to reflect differences in oligosaccharide content and/or processing, inasmuch as nucleotide sequence comparison between the GS and Z29 gB genes predicts no difference in the amino acid residue number but differences in the number of potential *N*-glycosylation sites (Chou & Marousek, 1992). The two co-variations detected in gB allow clustering of HHV-6 isolates into two non-overlapping groups, one including GS and U1102, the other including Z29, the variant B strains Hashimoto and SF, and CV. The latter isolate was found to be similar to Z29 with respect to two further parameters, the lack of reactivity to the variant A-specific MAb to gp82-105 and the diagnostic *Hin*dIII endonuclease restriction site. Thus, the two HHV-6 clusters identified by analysis of gB are concordant with segregation into variants A and B (Ablashi *et al.*, 1993). Altogether, earlier and current studies show that the two clusters share common proteins and epitopes, and may be differentiated antigenically with respect to at least four proteins. It seems likely that the list of proteins containing epitopes specific for each cluster will increase as antibodies to additional proteins become available. Previous studies on the reactivity of MAb to gp82-105 and to p180 and of MAb OHV1 did not establish whether the lack of reactivity reflected the lack of the reactive epitope or a total lack of shared epitopes in the protein. Here we show that the variations occur within a conserved protein. Inasmuch as gB is an important component in virus entry for other herpesviruses and is a virion component in HHV-6 (Cirone *et al.*, 1993), the differences between variant A and variant B gB might correlate with the reported differences in host range specificities.

A large body of evidence has accumulated indicating that HHV-6 isolates may be clustered into two groups. Although the high degree of homology between the two variants might lead to recombination between the two genomes, such recombinants have not been recorded to date. The isolation of viruses characterized for an antigenic repertoire typical of variant A strains and displaying a mixed restriction pattern relative to a single *Hin*dIII restriction site (Ablashi *et al.*, 1991; Dewhurst *et al.*, 1992) argues for a dual isolation from the same host rather than for recombinants formed either *in vivo* or *in vitro*. We propose that MAb 2D9 should be employed in conjunction with the other variant-specific MAbs as diagnostic reagents to monitor HHV-6 isolates for antigenic composition and to establish a firm correlation between antigenic composition and genomic polymorphism and *in vitro* growth properties. For such a correlation to be significant, the number of antibodies should be high enough to detect variations in several regions of the genome; in this context there is a particular need for development of antibodies specific to the variant B strain group.

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